Articles

A Partially Folded Intermediate during Tubulin Unfolding: Its Detection and Spectroscopic Characterization

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ABSTRACT: The unfolding reaction of the dimeric protein tubulin, isolated from goat brain, was studied using fluorescence and circular dichroism techniques. The unfolding of the tubulin dimer was found to be a two-step process at pH 7. The first step leads to the formation of an intermediate conformation, stable at around 1−2 M urea, followed by a second step that was due to unfolding of the intermediate state. At pH 3, the urea-induced biphasic unfolding profiles obtained at pH 7 became a one-step process indicating that a stable intermediate was also formed at this pH. The intermediate at pH 3 was more stable toward urea denaturation than that at pH 7. The intermediate state has about 60% secondary structure, partially exposed aromatic residues, and less tertiary structure as compared to the native state. Also, hydrophobic surfaces were more exposed in the intermediate than in the native or unfolded states. These results indicate that the intermediate state observed during tubulin unfolding is not only distinct from both the native and unfolded forms but also possesses some properties characteristic of a molten globule.

How a protein chain folds into its unique, biologically active conformation is one of the major problems of biochemistry today. Earlier, protein folding was generally believed to be a highly cooperative, two-state process in which only the native and completely unfolded states were significantly populated, without any measurable amount of intermediate states. However, recent studies (both equilibrium and kinetic) have shown the existence of stable intermediate conformational states for several proteins (Kim & Baldwin, 1982) that are different from their functional, native conformations. Structural and thermodyamic characterization of these partly folded intermediates is important for an understanding of the rules that govern protein folding (Baldwin, 1991; Dill & Shortle, 1991; Dobson, 1992).

Perhaps the best-characterized example of such a conformation is the molten globule state induced in several proteins by mild denaturing conditions, viz., moderate concentrations of urea/GdnHCl[1] (Kuwajima, 1989; Baldwin, 1991; Ptitsyn, 1992), by extremes of pH and ionic strength (Goto et al., 1990a,b), by high temperature, or by small alterations in their chemical structures under physiological conditions (Shortle & Meeker, 1989; Amir & Haas, 1988; Kuwajima, 1989). This partly folded intermediate is almost as compact as the native protein, with solvent-accessible hydrophobic regions (Ptitsyn, 1987). It has a high content of secondary structure, often comparable to or less than the native state, but has apparently no defined tertiary structure (Ewbank & Creighton, 1991). After the molten globule state was first described for the proteins α-lactalbumin (Dolgikh et al., 1981, 1985; Kuwajima, 1977; Kuwajima et al., 1985) and cytochrome *c* (Ohgushi & Wada, 1983), the existence of molten globule-like intermediates has been demonstrated by kinetic experiments with several other proteins. This suggests that the molten globule may be an obligatory intermediate in the folding pathway, although its exact significance in the mechanism of folding is still not understood (Konishi et al., 1982; Denton et al., 1982; Lynn et al., 1984). Consequently, it is important to study other proteins in order to prove the general nature of such states and ascertain their role in protein folding.

In this paper, we report our results on the unfolding of the cytoskeletal protein tubulin induced by urea and acidic pH. Tubulin, a 100-kDa protein, is composed of two nonidentical subunits of about 450 amino acid residues each (Krauhs et al., 1981; Ponstingl et al., 1981). Our work comprises the identification and characterization of an intermediate state during tubulin unfolding that is quite different from both the native and unfolded states.

EXPERIMENTAL PROCEDURES

Reagents and Buffers. The buffers used for spectroscopic studies contained 20 mM sodium phosphate, 1 mM EGTA, and 0.5 mM $MgCl_2$. To obtain buffers of different pH, the pH was adjusted to the desired value by adding appropriate amounts of HCl or NaOH to the above. Unfolding conditions were achieved by appropriate additions from 10 M urea, obtained from Aldrich Chemical Co., in the same buffers. Stock solutions of urea were prepared freshly on the day of use. 1,8-ANS was obtained from Molecular Probes. Its concentration was checked spectrophotometrically using an extinction coefficient of 6.8×10^3 M^{-1} cm^{-1} at 370 nm.

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[1] Abbreviations: GdnHCl, guanidine hydrochloride; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP, guanosine triphosphate; 1,8-ANS, 1-anilino-8-naphthalenesulfonate; CD, circular dichroism; [θ], mean residue ellipticity; λ_{max}, wavelength of maximum fluorescence emission; FPLC, fast protein liquid chromatography.

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PIPES, EGTA, and GTP used for isolation of tubulin were from Sigma Chemical Co.

Purification of Tubulin. Tubulin was isolated from goat brains by two cycles of temperature-dependent assembly and disassembly in buffer containing 50 mM PIPES, 1 mM EGTA, and 0.5 mM $MgCl_2$, pH 7.0, followed by two further cycles in 1 M glutamate buffer (Hamel & Lin, 1981). The purified tubulin, free from microtubule-associated proteins, was stored in aliquots at −70 °C. Protein concentrations were estimated by the method of Lowry et al. (1951).

Sample Preparations. For denaturation experiments, protein samples were incubated for at least 2 h at each urea concentration or pH prior to making spectroscopic measurements. This 2-h incubation was sufficient to attain equilibrium, as unfolding of tubulin was observed to be complete within 10 min under the conditions employed.

Spectroscopic Methods. The unfolding under different denaturing conditions were monitored spectroscopically by the following methods:

Fluorescence spectroscopic studies were done to detect changes in intrinsic protein fluorescence on a Hitachi F3000 fluorescence spectrophotometer. Excitation was at 280 nm and emission spectra were recorded in the 300−400-nm wavelength range. For experiments involving the binding of 1,8-ANS to tubulin, excitation of the drug was done at 350 nm and relative emission intensity was monitored at 480 nm, 15 min after addition of the drug to the protein samples. The excitation and emission bandwidths were fixed at 5 nm each and a 0.4-cm path-length quartz cuvette was used for all measurements. The spectrum of each sample was corrected by subtraction of the buffer alone. Protein concentrations were 1 μM in all cases, unless otherwise mentioned.

Circular dichroism studies were done on a Jasco J600 spectropolarimeter. Secondary structure was monitored in the 200−260-nm wavelength region using a cell of path length 0.1 cm. Tertiary structure was monitored in the near UV region, i.e., the 250−320-nm wavelength range, using a 10-cm path-length cell. A spectral bandwidth of 1 nm and a time constant of 2 s were used for these measurements. Protein concentrations used was 1 μM in all cases, and each spectrum was recorded as an average of 6 scans. All measurements were made at 25 °C.

Data Analysis. To compare the transitions detected at different conditions, each unfolding curve was normalized to the apparent fraction of the unfolded form, f_u, using the following relation:

$$f_u = \frac{P_{obs} - P_{nat}}{P_{unf} - P_{nat}}$$

where P_{obs} is the value of the optical parameter being monitored (ratio of fluorescence intensity at 333 nm to that at 355 nm, for fluorescence studies) at a given denaturing condition and P_{nat} and P_{unf} are the respective values for the native and unfolded states. Mean residue ellipticities at 220 nm were calculated from the observed values of ellipticity using a mean residue weight of 111.

Statistical Analysis. The data obtained for unfolding of tubulin in urea from fluorescence and CD measurements were analyzed using a five-parameter, nonlinear, least-squares fit program. The parameters were systematically varied over a given range and a best-fit curve was obtained with those parameters that gave minimum χ^2 value. The midpoint of each transition was then calculated from the best-fit parameters.

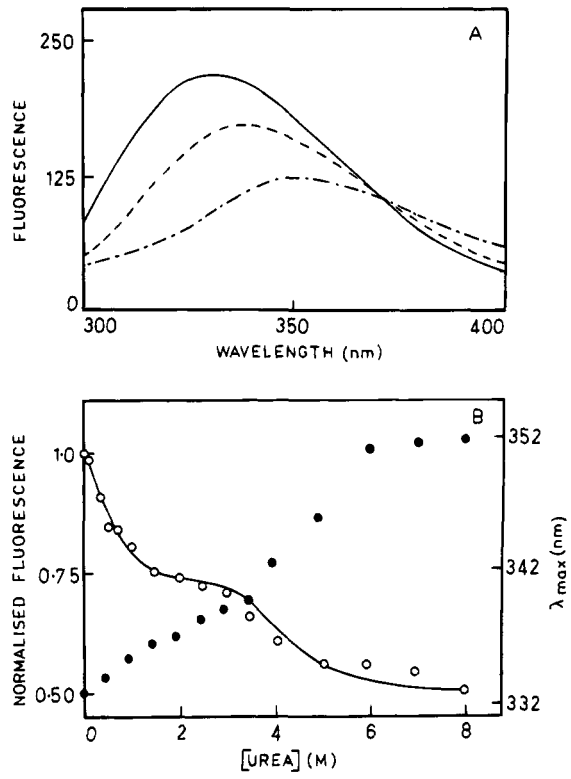

FIGURE 1: Equilibrium unfolding of tubulin in presence of urea. (A) Fluorescence emission spectra on excitation at 280 nm. Tubulin was 1 μM in 20 mM sodium phosphate buffer, pH 7, and the spectra were recorded in the absence of urea (—) and in the presence of 2 M (- - -) and 8 M (- • -) urea. (B) Urea dependence of tryptophan fluorescence intensity at 333 nm (○) and λ_{max} (●). The fluorescence values were normalized with respect to that in the absence of urea. The solid line through the F_{333} values represents the best-fit curve. Excitation and emission band-passes were 5 nm each.

RESULTS

Unfolding Detected by Fluorescence. Tubulin contains eight tryptophan residues, four each in each of the two subunits. On excitation at 280 nm, the wavelength of maximum emission of the tubulin tryptophans is 333 nm. The fluorescence spectra shifted progressively toward red and the fluorescence was quenched as the protein was exposed gradually to increasing concentrations of urea (Figure 1A). At 8 M urea, where tubulin is completely unfolded, the λ_{max} was 353 nm and the fluorescence intensity at 333 nm was quenched to 45−50% of its initial value in absence of urea. The fluorescence intensity at 333 nm in different urea concentrations was normalized with respect to that for the native protein and plotted against the respective urea concentrations (Figure 1B). The overall process was observed to be biphasic and the second transition starts at around 3 M urea, where the λ_{max} of the protein was 339 nm. The fluorescence at 333 nm was quenched by 20−25% of its initial value during the first transition. The second transition manifests a fairly sharp change in λ_{max} from 339 to 353 nm, where approximately 25% of the total fluorescence quenching occurs.

In solution, tubulin exists in an equilibrium between its monomeric and dimeric forms. The monomer ⇌ dimer

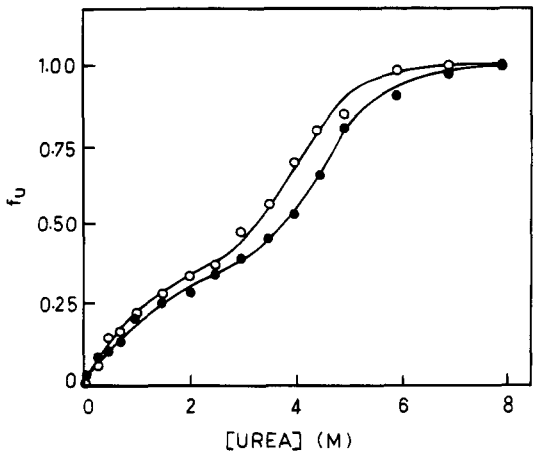

FIGURE 2: Fraction of protein unfolded (f_u) as a function of urea concentration. f_u was calculated from the ratio of fluorescence intensity at 333 nm to that at 355 nm. Tubulin concentrations were 0.1 μM (○) and 2 μM (●).

Table 1: Dependence of Midpoints of the Two Urea Denaturation Transitions, Detected by Fluorescence, on Tubulin Concentration

	urea concn (M) for half-maximal change during	
protein concn (μM)	transition 1	transition 2
0.1	0.85 ± 0.03	3.94 ± 0.2
2	0.92 ± 0.04	4.54 ± 0.01

equilibrium can easily be shifted to the left by dilution of the protein (K_d = 0.72 μM) (Panda et al., 1992). The unfolding of goat brain tubulin in urea was monitored at different protein concentrations (from 0.1 μM, corresponding to 10% dimer, to 2 μM, corresponding to 55% dimer) to examine whether subunit dissociation affects the observed biphasic unfolding pattern in any way. Figure 2 shows the urea dependence of the fraction unfolded at two different concentrations of tubulin. That unfolding proceeds via a two-step process is also evident from these plots. This suggests that the first transition probably signifies partial unfolding of the native state into an intermediate state, stable at around 2 M urea. Here the λ_{max} is 337 nm, indicating that solvent exposure of the tryptophans is somewhat greater than in the native state. The second transition is probably due to disruption of residual tertiary structure into the totally unfolded protein (confirmed by the λ_{max} value in 8 M urea). The values of the midpoints of the two transitions at different protein concentrations are shown in Table 1. The stability of tubulin to denaturation probably increases with higher concentration as suggested by the slight differences in unfolding patterns caused by the monomer and dimer.

To demonstrate that the observed unfolding pattern was not an experimental artifact caused by protein aggregation at mild urea concentrations, dependence of tryptophan fluorescence intensity of tubulin on its concentration was studied both in the absence and in the presence of 2 M urea (data not shown). The fluorescence at 333 nm showed linear dependence on tubulin concentration in the range studied, 0.1−2 μM. All measurements were made within the linear range of tubulin concentrations.

To determine whether any other solution conditions induce the intermediate state, the intrinsic protein fluorescence was measured in buffers of different pH in the range 2−7. No data were taken between pH 3.5 and 5.5 due to precipitation of the protein. The fluorescence value at each pH was

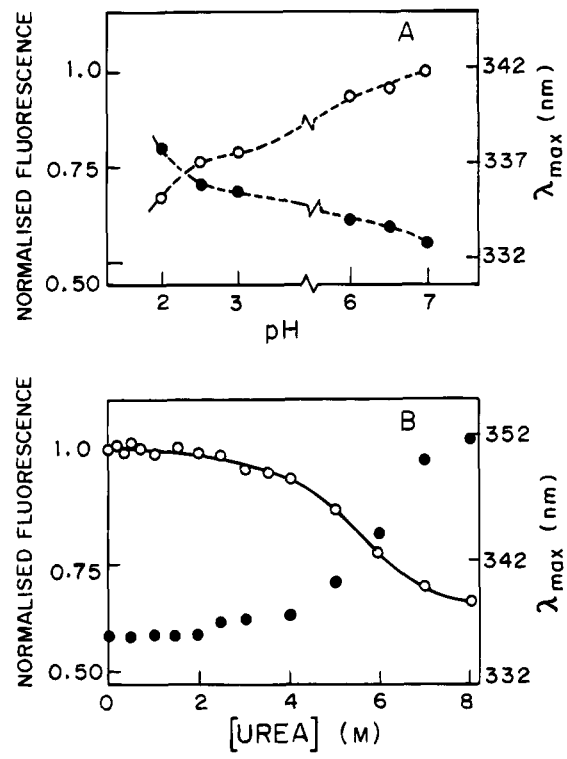

FIGURE 3: Variation of intrinsic protein fluorescence at 333 nm (○) and λ_{max} (●) with (A) pH and (B) urea at pH 3. Tubulin concentration used was 1 μM. The fluorescence values at each point were normalized with respect to that at pH 7 (for panel A) and that in the absence of urea at pH 3 (for panel B). The solid line through the fluorescence values in (B) is the best-fit curve.

normalized with respect to that at pH 7 and plotted against the respective pH (Figure 3A). The nature of the curve suggests the formation of an intermediate state around pH 3, where the fluorescence at 333 nm is quenched by 25% and the λ_{max} is 336 nm.

To confirm the existence of an intermediate state at pH 3, the tryptophan fluorescence at 333 nm was measured in different urea concentrations at pH 3 (Figure 3B). The fluorescence intensity and λ_{max} remains nearly invariant up to 3.5 M urea, followed by a single transition where the fluorescence is quenched by almost 25% of its initial value at pH 3 without urea and the λ_{max} is shifted to 352 nm. Thus, the biphasic unfolding pattern in urea at pH 7 becomes a one-step process in pH 3. This may be due to the fact that, at pH 3, the protein is already in the intermediate state that is converted to the unfolded state by urea via a single transition which has its midpoint at approximately 5.7 M urea.

Unfolding Detected by CD: Far UV CD. The far UV CD spectrum of native tubulin is typical of a protein with relatively low α-helix content, that of tubulin being approximately 20−25% (Lee et al., 1978). It consists of a strong negative band in the 215−222-nm region and a weaker one at 208 nm.

The negative CD band in the far UV region becomes less pronounced with increasing urea concentration or lowering of pH. The mean residue ellipticities at 220 nm ($[\theta]_{220}$) were normalized with respect to that in the absence of urea at pH 7 and plotted against the respective urea concentration (Figure 4A). The variation of $[\theta]_{220}$ with urea concentration followed a biphasic pattern. In the first phase, the midpoint of which is at approximately 0.3 M urea for 1 μM tubulin,

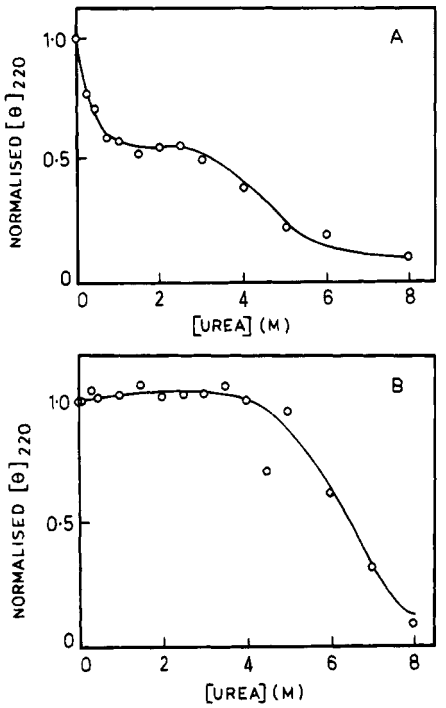

FIGURE 4: Dependence of mean residue ellipticity at 220 nm on urea at (A) pH 7 and (B) pH 3. The ellipticity values were normalized with respect to that in the absence of urea. The solid lines represent the best-fit curves. Protein concentration was 1 μM and a 1-cm path-length cell was used.

$[\theta]_{220}$ is decreased by almost 45% of that in the absence of urea. It then remains almost unperturbed from 0.75 to 2.5 M urea, indicating that an intermediate state is formed in these denaturing conditions. Further increase in the urea concentration leads to loss of negative ellipticity until there is almost total loss of structure of the CD band in 8 M urea. The midpoint of the second transition is at 4.5 urea for 1 μM tubulin.

The mean residue ellipticity at 220 nm also changes in a pH-dependent manner (data not shown). At pH 3.0, the mean residue ellipticity at 220 nm is about 58% of that at pH 7.0 and these CD spectra are almost identical with those obtained at 1−2 M urea. Further decrease in $[\theta]_{220}$ occurs when the pH is lowered below 2.5. The mean residue ellipticity at 220 nm was also measured in different urea concentrations at pH 3.0 (Figure 4B). It was found that the $[\theta]_{220}$ values remained almost unchanged up to 4 M urea at pH 3, followed by a single transition, with midpoint at 6 M urea, leading to a complete loss of negative ellipticity at 220 nm. This confirms the formation of a stable intermediate at pH 3 that unfolds in urea by a one-step process.

These far UV CD results confirm the findings obtained from fluorescence measurements, i.e., an intermediate state is formed in 1−2 M urea, pH 7, and at pH 3. Both the urea-induced and pH-induced intermediate states of tubulin possess considerable secondary structure, their formation being accompanied by a 42−45% loss in $[\theta]_{220}$.

Near UV CD. The CD spectrum of tubulin was measured in the 250−320-nm range as a probe to monitor changes in the environment of tryptophan and tyrosine side chains, caused by urea and low pH (Figure 5). In the absence of denaturant, the spectrum shows a broad negative band with a double minimum at 278 and 285 nm. In 1−2 M urea, there is a dramatic decrease in negative ellipticity in this

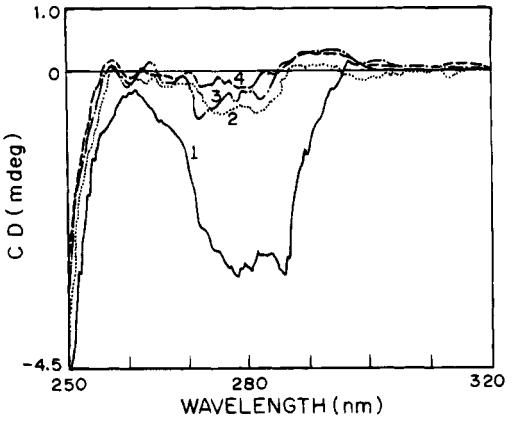

FIGURE 5: Near UV (250−320 nm) CD spectrum of tubulin. The spectra were recorded in the absence of urea at pH 7 (spectrum 1) and pH 3 (spectrum 2) and in the presence of 2 M (spectrum 3) and 8 M (spectrum 4) urea at pH 7. Protein concentration was 1 μM, and a 10-cm path-length cell was used for these measurements.

region leading to a weak, featureless spectrum, comparable to that of the unfolded protein in 8 M urea. A similar, practically featureless spectrum was also obtained in pH 3.0. This indicates that the aromatic residues in the intermediate state are no longer in an asymmetric environment, i.e., a substantial loss in tertiary contacts has occurred in denaturing conditions which are insuffficient to completely disrupt secondary structure. These mild denaturing conditions are seen to affect the CD band at 280 nm to a greater extent than that in the 290−295-nm region. Thus, probably the average tyrosine environment undergoes a greater perturbation compared to tryptophans in the intermediate state.

The convergence of all CD spectra to essentially zero ellipticity at 250 nm (for far UV) and 300 nm (for near UV) suggests that a differential light scattering artifact was absent under the conditions used. Absence of any significant light scattering was also confirmed by examining the baselines of the UV absorbance spectra in the 300−350-nm wavelength region (data not shown).

ANS Binding. The hydrophobic marker drug 1,8-ANS shows only a weak fluorescence when free in aqueous solutions. Its fluorescence is markedly increased in nonpolar environments, like hydrated hydrophobic surfaces in proteins, and a blue shift in emission maximum occurs. The binding of ANS to tubulin was measured as a function of urea concentration and pH by recording ANS fluorescence intensity and λ_{max} at room temperature. Figure 6A shows that the fluorescence at 480 nm is increased almost 1.5 times upon binding to tubulin in presence of 0.2 M urea compared to that in the absence of urea. Further increase in urea concentration to 1.5−2 M results in a 2−2.5-fold enhancement in fluorescence. The wavelength of emission maximum of ANS also undergoes a blue shift from 487 to 481 nm upon binding to the intermediate state. This is followed by a red shift to 508 nm and a drastic decrease in fluorescence intensity as the protein becomes unfolded in higher urea concentrations.

The fluorescence of ANS upon binding to tubulin is also enhanced in a pH-dependent manner (Figure 6B). The relative fluorescence intensity of ANS at 480 nm begins to increase upon lowering the pH from 7.0, until it is almost 6−7-fold enhanced at around pH 3.0. This is accompanied by a 11-nm blue shift in emission λ_{max}. Below pH 2.5, the fluorescence intensity decreases while the λ_{max} shifts toward

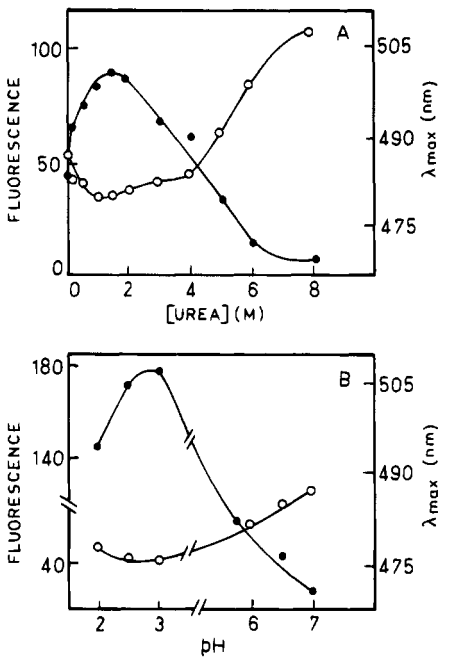

FIGURE 6: (A) Effect of urea at pH 7 and (B) effect of pH on ANS fluorescence in the presence of tubulin. ANS fluorescence intensities at 480 nm (●) and λ_{max} (○) values are shown. Incubation of the protein (1 μM) with 1,8-ANS (10 μM) was for 15 min at 25 °C, and the excitation wavelength was 350 nm.

red, indicating further unfolding of the intermediate state. Such enhancement of fluorescence intensity upon binding of ANS to tubulin, coupled with a blue shift in emission maximum in mild urea concentrations or low pH, suggest that partial unfolding of the protein results in exposure of solvent-accessible hydrophobic surfaces which makes ANS binding to the intermediate state more facile compared to the native state.

To determine whether ANS is itself responsible for any conformational changes in the protein, far UV CD spectra of tubulin were taken both in the absence and in the presence of ANS (data not shown). The CD spectra were found to be identical, indicating that the concentration of ANS (10 μM) used in these studies does not induce any structural change in tubulin (1 μM) within the 15-min incubation time.

DISCUSSION

Equilibrium denaturation experiments on several proteins have revealed the existence of partly folded intermediate states that can be adopted by a polypeptide chain under certain conditions. Such states have been induced in vitro at low pH, high temperature, and high ionic strength as well as by low concentrations of structure-perturbing agents such as urea and guanidine hydrochloride. Interest in such intermediates is strong, as they are thought to resemble kinetic intermediates formed early in the folding pathway (Kim & Baldwin, 1990; Udgaonkar & Baldwin, 1988). Early folding intermediates have been detected for several globular proteins including lysozyme, α-lactalbumin, and parvalbumin (Kuwajima et al., 1985, 1987, 1988). These intermediates have appreciable amount of secondary structure but very little tertiary structure and are popularly known as "molten globules".

An intermediate in the thermal denaturation of tubulin has already been reported (Mozo-Villarias et al., 1991). Recently, Sackett et al. (1994) reported differential urea sensitivities of the various properties of tubulin. These include the ability to polymerize into microtubules, GTPase activity, proteolysis by trypsin and chymotrypsin, and binding of the drugs ANS and 2-methoxy-5-(2′,3′,4′-trimethoxyphenyl)tropone. These functions can be differentially modified by low (0.1−1.0 M) urea concentrations, and such urea-induced modifications are stable over time periods of minutes to hours. These results have been explained on the basis of the existence of restricted regions in the protein, each of which is associated with a functional property having its own urea sensitivity. These low urea concentrations do not induce significant changes in tryptophan fluorescence.

We observed that the unfolding of goat brain tubulin in the presence of urea proceeds via a two-step process: the first step, which signifies formation of an intermediate state, followed by a second transition that manifests unfolding of the intermediate state. We have identified and characterized this intermediate state, observed at low concentrations of urea, by measuring the changes in intrinsic tryptophan fluorescence, the binding of the hydrophobic fluorescence probe ANS, and the far UV and near UV CD. A stable, partially unfolded conformation resembling the intermediate state obtained at low urea was also observed at pH around 3.0.

The λ_{max} of tryptophan fluorescence emission for the intermediate state (336−337 nm) is between those for the native (333 nm) and unfolded (353 nm) states. Thus, at least some of the hydrophobic aromatic residues of tubulin are more hydrated in the intermediate state than in the native form. Complete exposure to polar environment occurs only in the unfolded state in 8 M urea.

The far UV CD spectra shows that formation of the intermediate state of tubulin is accompanied by an approximately 42−45% decrease of mean residue ellipticity at 220 nm. This behavior is similar to that of the molten globule-like state of barstar which has about 40% less negative ellipticity in the far UV region compared to the native state (Khurana & Udgaonkar, 1994). On one hand, this may not be a true estimate of secondary structure content, as the mean residue ellipticity at 220 nm may be influenced by contributions from aromatic residues and sulfhydryl groups. On the other hand, the observed decrease in $[\theta]_{220}$ accompanying formation of the intermediate state may also be due to local unzippering of secondary structure in some highly exposed part of the protein. It is known that the carboxy-terminal tails of tubulin stay extended in the surrounding medium, at physiological pH (Sackett & Wolff, 1986). It is also known that the C-terminal amino acid sequences of both the α and β subunits of tubulin have high α-helix potential (Ponstingl et al., 1983). So it is not difficult to visualize an intermediate state in mild denaturing conditions where any secondary structure in the highly exposed tails of tubulin is melted, while that in the main frame of the molecule remains unaltered.

The environment of the aromatic residues in the intermediate state is quite different from those in the native state as evident from the near UV CD spectra. The dearth of structure discernable in the near UV CD spectrum of the intermediate state in contrast to that seen for the native state is a characteristic reminiscent of the "molten globule" state.

The drug 1,8-ANS is extensively used as a probe to measure the hydrophobicity of a protein surface. It has been

widely used to detect the formation of molten globule-like intermediates in the folding pathways of several proteins (Ptitsyn et al., 1990). An enhancement of ANS fluorescence intensity and a blue shift in emission maximum were observed when the drug was bound to the intermediate state relative to native tubulin. This indicates that hydrated hydrophobic surfaces were more exposed in the partially unfolded intermediate state than in the native state.

Thus the intrinsic tryptophan fluorescence, CD, and the increased hydration of hydrophobic residues as shown by ANS binding studies all indicate that the intermediate state of tubulin observed at low urea concentration and low pH is not only distinct from its native state but also possesses several distinct features that characterize a molten globule.

FPLC studies were carried out in this laboratory to assess the compactness of the intermediate state of tubulin by comparison of its Stokes radius with that of the native state (data not shown). The Stokes radius of the native, dimeric protein in the absence of urea was found to be 50 Å, while that of the unfolded monomer in 8 M urea was 65.7 Å. In 2 M urea, an early eluting species, corresponding to a Stokes radius of 98.2 Å, was observed in addition to a small fraction of Stokes radius 56.7 Å. On lowering the protein concentration, the population of the early eluting species was significantly reduced. This suggests that partial unfolding of the protein at mild denaturing conditions leads to exposure of hydrophobic surfaces that interact intermolecularly to form small aggregates. Aggregation appears to be a characteristic of the partially unfolded state. Aggregation at intermediate denaturing conditions has been reported for the TGFα–*Pseudomonas* exotoxin hybrid protein (O'Brien Gress et al., 1994) and porcine growth hormone (Bastiras & Wallace, 1992). Self-association via partially folded intermediate has also been observed for human growth hormone (DeFelippis et al., 1993) and acidic fibroblast growth factor (Mach et al., 1993). In our case, while aggregation of the intermediate state does not interfere with spectroscopic measurements, it makes any hydrodynamic studies difficult. However, it appears that the intermediate state has a Stokes radius in between those of the native and unfolded states. A molten globule state of hsp70 whose hydrodynamic size increases progressively with increasing urea concentration has been reported (Palleros et al., 1993). An idea of the compactness of the intermediate state of tubulin can be obtained from the work of Sackett et al. (1994). They observed that at low concentrations of urea (1.5 M) the accessibility of the protein to proteases like trypsin and chymotrypsin is considerably reduced compared to the native state while at higher concentrations of urea the rate of proteolysis increases with extensive unfolding. This suggests that low concentrations of urea might lead to an overall "tightening" of tubulin structure, i.e., compactness is retained.

The degree of similarity between the intermediate states induced by urea and low pH is not very clear. Their tryptophan emission maxima are comparable, 337 nm for the former and 336 nm for the latter. However, ANS binding experiments show an almost 6-fold increase in fluorescence quantum yield with a 11-nm blue shift in λ_{max} for the acid-induced state, while the urea-induced state shows only a 2-fold increase in quantum yield and a 6-nm blue shift in λ_{max}. The acid-induced intermediate unfolds at a higher concentration of denaturant at pH 3 than the urea-induced state at pH 7, as determined from both fluorescence and circular dichroism studies. From fluorescence studies it was seen that for 1 μM tubulin the midpoint of the second transition at pH 7, which signifies unfolding of the intermediate state, is at 4.3 M urea, while that of the single transition at pH 3 is at 5.7 M urea. For CD-monitored denaturation, the midpoint of the second transition at pH 7 is at 4.5 M urea, while that at pH 3 is at 6 M urea. Thus the acid form at pH 3 is probably more stable than the intermediate state induced by urea at pH 7. Similar enhancement of stability was also observed in acid-induced molten globule-like state of barstar (Khurana & Udgaonkar, 1994) when compared with its native form at pH 7. On the contrary, it has been reported that the acid-induced molten globule state of carbonic anhydrase B melts at a lower concentration of denaturant than the native state (Ptitsyn, 1992).

Although the CD spectrum of the acid-induced intermediate shows no well-defined tertiary structure, the cooperative unfolding caused by urea at pH 3 indicates that it may be stabilized by tertiary interactions. Such behavior has also been observed for the acid-denatured conformations of the MAK33 antibody (Buchner et al., 1991) and barstar (Khurana & Udgaonkar, 1994), but no definite evidence had been obtained of specific tertiary contacts.

In conclusion, we have shown that goat brain tubulin exists in an intermediate conformation that resembles a molten globule state, in the presence of low urea concentrations and at low pH. We have not determined the functional properties of tubulin under these conditions. According to recent reports, properties of tubulin which are affected by low urea concentrations are its polymerization, GTPase activity, binding of the drugs ANS and 2-methoxy-5-(2′,3′,4′–trimethoxyphenyl)tropone, protease sensitivity, etc. (Sackett et al., 1994). These various properties of tubulin, unrelated to each other, are expected to be distributed in different areas of tubulin. It is possible that the presence of low concentrations of urea, which partially unfold tubulin, affects the secondary and tertiary structure of these areas. It remains to be seen whether tubulin can be refolded reversibly to its native state with reference to both structural and functional properties.

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